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The comparative study on the cuticle micro-morphology of *Pilgerodendron uviferum* (Cupressaceae) and its relatives

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Abstract The taxonomic position of the monotypic conifer *Pilgerodendron uviferum*, an endemic species in southern South America, has been disputed on the basis of phylogenetic analysis using molecular data. These results suggest that *P. uviferum* is a species of *Libocedrus*. Phylogenetic analysis using morphological data appears to uphold its separate status. Characters of leaf cuticles are investigated and compared between four related genera including *Pilgerodendron* and further distinctions between these are reported. The merits of these conflicting data sets for the taxonomy of the genus *Libocedrus* s.l. are discussed. It is concluded that the evidence for a merger of *Pilgerodendron* with *Libocedrus* is weak and that both morphology and biogeography support the separation of the two genera.

Key words Cupressaceae, *Pilgerodendron uviferum*, leaf cuticle, Florin ring.

The original description of this conifer (by D. Don in Lambert, 1828), *Pilgerodendron uviferum*, was not accompanied by an illustration and its author classified it in *Juniperus* L. naming it *J. uvifera* D. Don. Hooker (1844) described it as a new species *Thuja tetragona* Hook. but had some notion of its true identity: "Yet there is described by Mr. Don… a juniper, *J. uvifera* Lamb. Pin.;… and, were it not that the fruit is described as that of a true juniper, I should consider the two plants to be the same". We now know, of course, that there are no species of *Juniperus* native to South America. When Endlicher (1847) erected the genus *Libocedrus* he included among its three species *L. tetragona* (Hook.) Endl. which is now *Pilgerodendron uviferum* (D. Don) Florin. Florin (1930) corrected Hooker and Endlicher by taking up the earlier epithet *uvifera* and at the same time separated this taxon from *Libocedrus* and placed it in a new genus *Pilgerodendron*. This was accepted by Li (1953) in his reclassification of *Libocedrus* s.l. as a monospecific genus, a treatment which has since been accepted by numerous authors on the basis of several distinct morphological characters.

Gadek et al. (2000) conducted a phylogenetic analysis of Cupressaceae. They found a clade with the following taxa: *Libocedrus plumosa* (D. Don) Sargent (*L. yateensis* Guillaumin, *P. uviferum*) in which *L. yateensis* appeared to be more closely related to *Pilgerodendron* than it is to the other species of *Libocedrus*. This was the result of an analysis of combined *mat*K plus non-molecular data and there was >90% bootstrap support for this topology. Analysis of the *mat*K gene alone gave similar results. These results have cast doubts on the validity of the recognition of this taxon in its own genus. It is therefore necessary to investigate the evidence carefully, both for and against a

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separate status of *P. wiferum*. Thus, we here discuss the morphological characters which were used by earlier authors, e.g., Florin (1930, 1931), Li (1953), Page (1990), Farjon (2004) and others who have upheld its separate status. We will also critically look at the non-molecular characters used by Gadek et al. (2000) to see if they have included those that were found to be diagnostic by the earlier authors, and discuss the topology of their cladogram.

In order to discover additional morphological characters that may be informative, we have also investigated the leaf cuticles of three species from three genera that were formerly classified in *Libocedrus* s.l. and the genus *Austrocedrus* (see Table 1). These taxa formed a well-supported clade in a phylogenetic analysis of Cupressaceae based on morphology by Xiang and Farjon (2003) and Farjon (2004). The characters found will be presented and used in the discussion about relationships.

1 Material and Methods

Fresh foliage of *Austrocedrus chilensis*, *Libocedrus plumosa* and *Pilgerodendron uviferum* were collected in August 2001 from the Royal Botanic Gardens, Kew. The leaves of *Papuacedrus papuana* were taken from herbarium specimen. Each species of the four related genera was investigated. Information on the species, including name, source and geographic distribution, is presented in Table 1. Leaves were taken from comparable portions of the shoot in terms of morphology and stage of foliage development in the different species. Leaves were macerated in Jeffrey's Solution at room temperature for about 24 h. Isolated cuticles were washed thoroughly in water and dehydrated in a series of 50%, 75%, 95% and 100% ethanol and mounted on stubs with double-sided adhesive tape. Stubs were sputter-coated with gold-palladium for three minutes using a Polaron sputter coater and examined on a Hitachi S-2400 scanning electron microscope (SEM) at 18 kV and a standard tilt of 30°.

Thirteen features of the leaf cuticle were used to construct the character matrix for similarity analysis of four species of four related genera in Cupressaceae (Tables 2, 3). The analyses were run using PAUP* (Swofford, 1999). Clustering was achieved using UPGMA.

Table 1 Name, source and distribution of plant materials

Species	Source	Geographic distribution
Austrocedrus chilensis (D. Don) Pic. Serm. & Bizzarri	Kew Gardens, Acc. No. 1956-48702	South America
Libocedrus plumosa (D. Don) Sargent	Kew Gardens, Acc. No. 1990-8108	New Zealand
Papuacedrus papuana (F. Mueller) H. L. Li	T. G. Hartley 12912 in Kew Herbarium	New Guinea
Pilgerodendron uviferum (D. Don) Florin	Kew Gardens, Acc. No. 1988-3603	South America

2 Results

2.1 The micro-morphology of the leaf cuticle

2.1.1 *Austrocedrus chilensis* (Figs. 14, 16, 18, 20)

Adaxial cuticle, internal surface: Flanges are shallow and frequently interrupted. The edges of the flanges are coarsely granular. The sculpture of the periclinal surface of epidermal cells is coarsely granular.

Adaxial cuticle, external surface: The Florin ring has a low ridge and is lobed, uninterrupted, and without a peripheral groove.

Abaxial cuticles internal surface: The characters are similar to those of adaxial cuticle internal surface except flanges are stronger than those of adaxial cuticle.

Table 2 Characters and character states of leaf cuticles of four species of the tribe Libocedreae (Cupressaceae)

1. Abaxial stomata (absent = 0, present = 1)
2. Florin ring (absent = 0, present = 1)
3. Papillae in stomatal areas (absent = 0, present = 1)
4. Pits in the outer surface of non-stomatal areas (absent = 0, present = 1)
5. Sculpture of anticlinal flange apex of non-stomatal cells on the abaxial side (coarsely granular = 0, finely granular = 1)
6. anticlinal flange depth of non-stomatal cells on the abaxial side (shallow = 0, deep = 1)
7. Continuity of flange of non-stomatal cells on the adaxial side (continuous = 0, interrupted = 1)
8. Sculpture of periclinal surface of non-stomatal cells on the abaxial side (coarsely granular = 0, finely granular = 1)
9. Sculpture of periclinal surface of non-stomatal cells on the adaxial side (coarsely granular = 0, finely granular = 1)
10. Sculpture of periclinal surface of subsidiary cells (coarsely granular = 0, finely granular = 1)
11. Crystal tubercles on the periclinal surface of non-stomatal cells (absent = 0, present = 1)
12. Pits in the periclinal surface of subsidiary cells (absent = 0, present = 1)
13. Slots in the periclinal surface of subsidiary cells (absent = 0, present = 1)

Table 3 Character state values of 13 characters for four species of Cupressaceae used in a similarity analysis

Species	Character*												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Austrocedrus chilensis	0	1	0	0	0	0	1	0	1	1	0	0	1
Papuacedrus papuana	1	1	0	0	0	0	1	0	1	1	0	1	0
Pilgerodendron uviferum	0	0	0	1	1	1	0	0	0	1	1	0	1
Libocedrus plumosa	0	1	1	1	1	1	0	0	0	1	1	0	1

* Characters 1 – 13 are the same as stated in Table 1.

Abaxial cuticle, external surface : The Florin ring has an intermediate elevation, but it is not obvious like *Libocedrus plumosa* 's and is lobed, uninterrupted, with an even groove. Papillae are associated with stomata.

2.1.2 *Libocedrus plumosa* (Figs. 2, 6, 11 – 13, 15, 17, 19)

Adaxial cuticle, internal surface : There are crystal tubercles in the periclinal surface of epidermal cells. The crystal tubercles are embedded in a coarsely granular surface. The sculpture of the crystal tubercles is finely granular compared with that of other parts of the epidermal cells.

Adaxial cuticle, external surface : no SEM image was available for observation.

Abaxial cuticle, internal surface : The stomata form two bands along the margin of the leaf and the epidermal cells between them are rectangular, elongate along the long axis of the leaf. There are pits or furrows in the periclinal surface of subsidiary cells. There are crystal tubercles (pointed out by arrowhead in Fig. 19) in the periclinal surface of epidermal cells in non-stomata areas.

Abaxial cuticle, external surface : The Florin ring has an intermediate elevation, and is lobed, uninterrupted, with a peripheral groove. Papillae are associated with stomata.

2.1.3 *Papuacedrus papuana* (Figs. 3, 7, 8, 10)

Adaxial cuticle, internal surface : The flanges are shallow. The edges of the flanges are coarsely granular. The sculpture of the periclinal surface is coarsely granular. There are large pits along the flanges of subsidiary cells. Crystal tubercles are absent.

Adaxial surface, external surface : The stomata are scattered near edges of leaves, the Florin rings are prominent. The outlines of the epidermal cells are not clear. Pits (pointed out by arrowhead in Fig. 7) are concentrated in stomatal areas.

Abaxial cuticle, internal surface : The flanges are shallow and straight. The stomata are

Figs. 1 – 6. Characters of the external surface (1 – 4) and the internal surface (5, 6) of leaf cuticles in *Pilgerodendron uviferum* (1, 4, 5), *Papuacedrus papuana* (3) and *Libocedrus plumosa* (2, 6). **1.** Stomata without a Florin ring. **2, 3.** Stomata with Florin rings. **4.** Non-stomatal outer surface with small pits in the epidermal cells. **5.** Stomatal region with finely granular epidermal and subsidiary cells. **6.** Stomatal region with coarsely granular epidermal and finely granular subsidiary cells.

distributed in the middle part of the leaf. The boundary of the subsidiary cells is not distinct. There are some pits in the periclinal surface of subsidiary cells. The sculpture of the periclinal surface of subsidiary cells is finely granular compared with that of cells of the non-stomatal zone. Crystal tubercles are absent.

Figs. 7 – 12. Characters of the internal surface of leaf cuticles in *Papuacedrus papuana* (7, 8, 10), *Pilgerodendron uviferum* (9) and *Libocedrus plumosa* (11, 12). **7.** Stomatal region of the adaxial surface, with large pits. **8.** Stomatal region on the abaxial surface. **9.** Non-stomatal region, with crystal tubercles in cells with steep flanges. **10.** Non-stomatal region, with shallow flanges and absence of tubercles. **11.** Non-stomatal region, with steep flanges and coarsely granular surface. **12.** Non-stomatal region, with crystal tubercles embedded in coarsely granular surface.

Abaxial cuticle, external surface : In the stomatal areas, the Florin rings are prominent and steep sided. The periclinal surfaces of the guard cells are very smooth. The outlines of the epidermal cells are not clear. Pits on the surface of epidermal cells and papillae are absent.

2.1.4 *Pilgerodendron uviferum* (Figs. 1, 4, 5, 9)

The characters of the abaxial side and the adaxial side of the leaves are similar.

Abaxial cuticle, internal surface : stomata areas : Generally there are four or five subsidiary cells to each stoma. Usually subsidiary cells are shared. The subsidiary cells are polygonal, they are arranged radially and do not form a circle or ellipse. There are deep furrows in the periclinal surface of the subsidiary cells. The edge of the flange of subsidiary cells is thin and serrated. Crystal tubercles are absent in subsidiary cells. The sculpture on the periclinal surface of subsidiary cells is finely granular compared with that of cells of the non-stomatal zone.

Non-stomata areas : The sculpture on the periclinal surface of subsidiary cells is coarsely granular. The flanges are medium thick and straight. The edge of the flange is round and smooth. Crystal tubercles (pointed out by arrowhead in Fig. 9) are present in non-stomata areas near the leaf margin but absent in other areas.

Abaxial cuticle, external surface : Because of the heavy wax, the outlines of the epidermal cells are not clear. Only the stomatal pits were observed. There are no Florin ring papillae around the stomatal slots (pointed out by arrowhead in Fig. 5).

2.2 Comparison of *Pilgerodendron uviferum* with some of its nearest relatives

In Table 2, 13 epidermal characters and their states are listed and in Table 3 the character states for *Pilgerodendron uviferum* and three related taxa are presented. The UPGMA diagram (Fig. 21) shows two clusters on the basis of these data, one containing *Austrocedrus chilensis* and *Papuacedrus papuana*, the other with *Libocedrus plumosa* and *Pilgerodendron uviferum*. A Florin ring is present in all taxa except *Pilgerodendron uviferum*. The latter taxon also differs from *Libocedrus plumosa* in the absence of papillae in the stomatal areas, but in all other characters it is similar. In *Libocedrus plumosa*, surface prominences occur. The surface sculpture is conspicuously smoother or finer on the prominences than on the general periclinal surface. This character has not been included in the data matrix as it was difficult to code as a presence/absence character. The differences between *Pilgerodendron* and the two genera *Austrocedrus* and *Papuacedrus* are more numerous, as expressed in the UPGMA diagram.

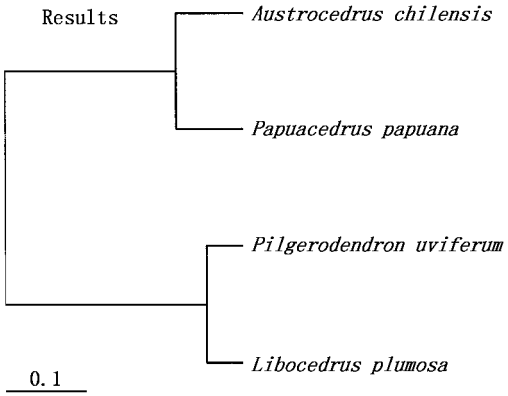

Fig. 21. UPGMA diagram showing clustering and similarity distances among four species of Cupressaceae based on 13 leaf cuticle characters.

3 Discussion

The leaf cuticles of conifers provide useful taxonomic characters (e.g. Florin, 1931 ; Stace, 1965 ; Oladele, 1983a, b ; Hu, 1986 ; Xiang & Fu, 1998) that can indicate phylogenetic relationships. However, the complexity of morphological evolution makes it highly unlikely that such a

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Figs. 13 – 20. Characters of the external surface (13 – 17) and the internal surface (18 – 20) of cuticles in *Libocedrus plumosa* (13, 15, 17, 19) and *Austrocedrus chilensis* (14, 16, 18, 20). **13, 14, 17.** Stomata with Florin rings. **15, 16.** Non-stomatal outer surface with small pits in the epidermal cell. **18.** Stomatal region with coarsely granular epidermal and subsidiary cells. **19.** Non-stomatal region, with crystal tubercles in cells. **20.** Non-stomatal region, with shallow flanges and absence of tubercles.

limited set of characters would constitute reliable evidence on its own. When put to the test, analysis of other sets of data may give incongruent results. A" total evidence "approach, while unachievable in a literal sense, is still the only one that gives results that are more robust and will provide a more probable hypothesis of phylogenetic relationships. The approach taken by Gadek et al. (2000) would seem a step in this direction as they have combined molecular data with morphological (" nonmolecular ") data. However, many if not most of these" nonmolecular "characters used in their analysis are not informative among the closely related taxa here analysed. Their characters 4 – 9 (wood anatomy) and 14 – 18 (leaf anatomy) are in this category (see also Quinn & Gadek, 1988). Other characters they used are missing in all taxa relevant to this paper and therefore uninformative. The morphological characters that do separate *Pilgerodendron* from *Libocedrus* were not included in their character set. The use of secondary chemistry (leaf terpenes etc.) as taxonomic characters is at best controversial. The molecular data that support inclusion of *Pilgerodendron* in *Libocedrus* are from a single gene, *mat*K and such limited data are increasingly considered insufficient and unreliable as well (Crawford, 2000). From such data it is premature to conclude that *Pilgerodendron* is a species of *Libocedrus*.

There are other than cuticular morphological characters that separate *Pilgerodendron* from *Libocedrus*, e.g., leaf dimorphism and distribution of stomata on leaf surfaces, as well as the number of microsporangia per microsporophyll, which is much higher in *Pilgerodendron*. A morphological analysis (Farjon, 2004) using 53 characters of Cupressaceae indicated that *Pilgerodendron* could be more closely related to *Papuacedrus* than to *Libocedrus*. With such conflicting results, a merger of these taxa is at least premature and in fact unhelpful to a better understanding of true relationships. The enormous geographical disjunction between them was a major argument for Li (1953) to split the component parts of *Libocedrus* s.l. Geography should not determine taxonomy. However, with the knowledge that we now have about the long history of the break-up of Gondwana and the consequent separation of these taxa (if they once formed an ancestral unity), which did not get where they are by dispersal, makes taxonomic separation more likely. We prefer to treat *Pilgerodendron* as a genus separate from *Libocedrus*, two Gondwanan conifers of ancient common ancestry now separated by the expanses of the southern ocean.

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Pilgerodendron uviferum（柏科）及其相关类群叶角质层微形态特征的比较研究

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摘要 在扫描电镜下观察了分布于南半球的柏科 Cupressaceae 单种属 *Pilgerodendron uviferum* 及其相关类群的代表种 *Austrocedrus chilensis*、*Libocedrus plumosa* 和 *Papuacedrus papuana* 叶角质层内外表面的微形态特征。发现 *Pilgerodendron uviferum* 叶角质层外表面光滑，不具 Florin 环，而其余 3 个属的代表种均有明显的 Florin 环；且仅 *L. plumosa* 叶角质层外表面有乳状突起。观察结果支持传统的观点，即将 *P. uviferum* 作为独立的属；不支持基于 DNA 分析的结果，即将 *P. uviferum* 归并到 *Libocedrus* 属。发现 *Papuacedrus papuana* 叶角质层外表面有许多明显的小凹陷，与其相对应的叶角质层内表面有凹陷的小狭缝，该性状以前未见报道。

关键词 柏科；*Pilgerodendron uviferum*；叶角质层；Florin 环